Transport of Pyridoxine and Pyridoxal 5'-Phosphate in Isolated Rat Liver Mitochondria*

Alec Lui, Lawrence Lumeng, and Ting-Kai Li

From the Veterans Administration Medical Center and the Indiana University School of Medicine, Indianapolis, Indiana 46223

(Received for publication, July 26, 1982)

The transport of [14C]pyridoxal-P and [14C]pyridoxine into isolated rat mitochondria was studied by centrifugal filtration. The incubation medium contained 20 mM 2-oxoglutarate and 10 mM inorganic phosphate to inhibit metabolism of pyridoxal-P by the mitochondria. The ratio of [14C]pyridoxine space to [3H]H2O space rapidly attained unity independent of the [14C]pyridoxine concentration in the medium and remained unchanged for up to 90 min of incubation. These data suggest simple passive diffusion for the transport of pyridoxine into the mitochondria. By contrast, the ratio of [14C]pyridoxal-P space to [3H]H2O space rose rapidly to exceed 1 in the first 15 min and continued to rise at a slower rate for as long as it was measured. The accumulation of [14C]pyridoxal-P was not decreased by inhibitors and uncouplers of oxidative phosphorylation. Fractionation of the mitochondria with digitonin revealed that 19 and 30 pmol of [14C]pyridoxal-P/mg of protein were taken up by the mitochondria at 15 and 60 min of incubation, respectively. Most of the uptake in the first 15 min occurred in the intermembrane space, whereas the largest increase of [14C]pyridoxal-P between 15 and 60 min of incubation appeared in the matrix fraction. Significant binding of the [14C]pyridoxal-P to proteins in the two compartments was demonstrated by gel filtration. These data indicate that pyridoxal-P can rapidly enter the intermembrane space of isolated mitochondria, but its penetration into the matrix occurs at a slower and more sustained rate (i.e. 9-16 pmol/h/mg of protein). It is concluded that the transport of pyridoxal-P into isolated rat liver mitochondria is energy-independent and is most consistent with passive diffusion facilitated by protein binding once this coenzyme enters the different compartments of the mitochondria.

A large number of vitamin B6-dependent reactions in liver are carried out within the mitochondria. In B6 sufficient rats, as much as 20% of the hepatic B6 content is located in the matrix compartment of this organelle, and it consists mainly of pyridoxal-P and pyridoxamine-P (1). Because the matrix compartment of liver mitochondria cannot synthesize these B6 coenzymes from their precursors, their supply must depend solely on transport from extramitochondrial sources. Recently, we have demonstrated in isolated hepatocytes that newly synthesized pyridoxal-P of cytosol is transported into mitochondria (1). In this study, we examine in more detail the nature of the transport process by incubating isolated rat liver mitochondria in the presence of radiolabeled pyridoxal-P.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats, weighing 200-300 g, were purchased from Harlan Industries, Inc., Indianapolis, IN. They were maintained on water and standard laboratory chow (Wayne Lab-Blox, Allied Mills, Inc., Chicago, IL) ad libitum until use. This laboratory diet contained 8.13 ppm of pyridoxine-HCl. [4,5-14C]Pyridoxine-HCl (18 mCi/mmol) was a gift from Hoffmann-LaRoche through the courtesy of Dr. Myron Brin. [14C]Pyridoxal was prepared from [14C]pyridoxine by oxidation with MnO2 and the former was then used for the synthesis of [14C]pyridoxal-P (2). [14C]H2O (0.25 mCi/g) and [U-14C]sucrose (1-5 mCi/mmol) were purchased from New England Nuclear. Silicone oil (AR 200) with a specific density of 1.040 was supplied by Wacker Chemie, Munich, West Germany. All other chemicals were purchased from Sigma. Digitonin was recrystallized in absolute alcohol before use.

Methods—Liver mitochondria were isolated according to the method previously described (1) and were incubated at 30°C. The standard incubation mixture contained isolated mitochondria (3 mg of protein/ml), 60 mM triethanolamine-HCl (pH 7.4), 10 mM K-phosphate, 105 mM KCl, 2.5 mM MgCl2, 3 mM ATP, 20 mM 2-oxoglutarate, 0.25 µCi/ml of [14C]H2O, and either 3 µM [14C]pyridoxal-P, 3 µM [14C]pyridoxine, or 0.15 µCi/ml [14C]sucrose. The intactness of isolated mitochondria was examined routinely by measuring their respiratory control ratios. The mean of the respiratory control ratios, when measured using the standard incubation mixture (without 2-oxoglutarate and the radioactive compounds) and 5 mg succinate as substrate, was 4.5.

Centrifugal filtration was employed for rapid separation of mitochondria from the incubation medium (3). The procedure was performed by transferring a 0.7-ml aliquot of the mitochondrial suspension to a 1.7-ml Eppendorf microtube which contained a layer of 0.4 ml of silicone oil on top of a bottom layer of 0.4 ml of 1.8 M HClO4. Centrifugation was carried out in an Eppendorf microcentrifuge (Model 5412) supplied with a fixed angle head for 60 s. After centrifugation, the radioactive content of the top layer containing the mitochondria-free supernatant medium was measured. The Eppendorf microtube was then punctured with a needle, and an aliquot of the HClO4 extract was aspirated into a syringe. A 0.3-ml portion of this aliquot was then used for radioisotopic counting.

The space or volume of distribution of [14C]pyridoxal-P, [14C]pyridoxine, [14C]sucrose, or [14C]H2O in the mitochondria was calculated by the following equation (3):

$$V_s = V_i \times \frac{dpm_m}{dpm_s}$$

where $V_s$ is the space or volume of distribution of the labeled substrate in µl/mg of mitochondrial protein, $V_i$ is the volume of the supernatant medium in µl, dpm$_s$ is the total disintegrations/min in the pellet released into the HClO4 layer, dpm$_m$ is the total disintegrations/min in the supernatant medium, and $m$ is the amount of mitochondria in milligrams of protein. The extent of accumulation of [14C]pyridoxal-P and [14C]pyridoxine by mitochondria was expressed as the ratio of [14C]vitamin space to [3H]H2O space.

Fractionation of the mitochondria with digitonin was performed by...
the method of Greenswalt (4). After incubation with \(^{14}\text{C}\)pyridoxal-P, mitochondria were centrifuged and washed three times at 0 °C before treatment with digitonin, 0.1 mg/mg of mitochondrial protein. Mitoplasts were then separated by centrifugation and washed once at 0 °C prior to sonication and separation into inner membrane and matrix fractions.

Chromatographic separation of free \(^{14}\text{C}\)pyridoxal-P from protein-bound \(^{14}\text{C}\)pyridoxal-P was performed by gel filtration at 4 °C. Sephadex G-25 resin was equilibrated with 10 mM K phosphate buffer (pH 7.4) and packed in a column (1.5 × 60 cm) to a height of 56 cm. The sample (2-5 ml) was applied to the column and eluted with the equilibration buffer. The effluent was collected in 3-ml fractions, and the absorbance at 280 nm and the radioactivity of each fraction were measured.

RESULTS

Uptake of Pyridoxal-P and Pyridoxine by Liver Mitochondria—Fig. 1 shows the uptake of \(^{14}\text{C}\)pyridoxal-P by isolated liver mitochondria incubated in the presence of 2-oxoglutarate and 10 mM Pi. When 3 \(\mu\)M \(^{14}\text{C}\)pyridoxine was added to the incubation mixture, this vitamer equilibrated rapidly across the mitochondrial membranes. The ratio of \(^{14}\text{C}\)pyridoxine space to \([3\text{H}]\)H\(_2\)O space attained unity in 1 min, and it remained unchanged for up to 90 min. Identical results were obtained when the \(^{14}\text{C}\)pyridoxine concentration was increased to 30 \(\mu\)M, 300 \(\mu\)M, and 3 mM (Fig. 2). These data indicate that pyridoxine enters the mitochondria effectively by passive diffusion and that the transport is clearly not a concentrative process.

Fig. 1 also depicts the transport of \(^{14}\text{C}\)pyridoxal-P into liver mitochondria as a function of time. When mitochondria were incubated in the presence of 3 \(\mu\)M to 3 mM \(^{14}\text{C}\)pyridoxal-P, the ratios of \(^{14}\text{C}\)pyridoxal-P space to water space exceeded unity within 1 min of incubation. Depending on the concentration of \(^{14}\text{C}\)pyridoxal-P, these ratios were 1.7 to 3.5 in 15 min and increased to 2.7 to 4.8 in 90 min. The kinetics of uptake, therefore, involved a rapid uptake phase followed by a slower phase which continued for as long as it was measured. These data indicate that pyridoxal-P transport by liver mitochondria is an accumulative process. No significant change of

the respiratory control ratio was observed when mitochondria were incubated in the presence of pyridoxal-P up to a concentration of 3 mM.

Fig. 2 shows the extent of accumulation of \(^{14}\text{C}\)pyridoxal-P by isolated mitochondria in relation to the concentration of this vitamer in the medium. After 60 min of incubation, the extent of accumulation expressed as the ratio of \(^{14}\text{C}\)pyridoxal-P space to \([3\text{H}]\)H\(_2\)O space was about 4.5 at 3 \(\mu\)M \(^{14}\text{C}\)pyridoxal-P and 1.9 at 3 mM.

Effect of Inhibitors and Uncouplers of Oxidative Phosphorylation on Pyridoxal-P Transport—Table I shows that none of the inhibitors and uncouplers of oxidative phosphorylation decreased the accumulation of \(^{14}\text{C}\)pyridoxal-P by isolated liver mitochondria. These data indicate that the uptake of pyridoxal-P into mitochondria is not dependent on energy derived from oxidative phosphorylation. It is of interest that both 2,4-dinitrophenol and carbonyl cyanide m-chlorophenylhydrazone increased the ratio of \(^{14}\text{C}\)pyridoxal-P space to \([3\text{H}]\)H\(_2\)O space at 15 min to almost twice of that measured in the presence of ATP. These uncouplers also increased the ratio of \(^{14}\text{C}\)sucrose space to \([3\text{H}]\)H\(_2\)O space (Fig. 3). It is possible that these agents increased the extent of penetration of both pyridoxal-P and sucrose into the matrix space under the conditions of the experiment.

Distribution of Accumulated \(^{14}\text{C}\)Pyridoxal-P in Mitochondrial Subfractions—Since pyridoxal-P transport measured by the ratio of \(^{14}\text{C}\)pyridoxal-P space to \([3\text{H}]\)H\(_2\)O space did not provide information as to where this coenzyme was sequestered, experiments were performed to determine the extent of penetration and binding of pyridoxal-P in the various compartments of liver mitochondria. Mitochondria were incubated in the presence of 3 \(\mu\)M \(^{14}\text{C}\)pyridoxal-P. At 15 and 60 min, an aliquot of the mitochondrial suspension was used to determine the amount of \(^{14}\text{C}\)pyridoxal-P taken up into the mitochondria and in the adherent medium by centrifugal filtration. Simultaneously, another aliquot of the mitochondrial suspension was centrifuged, washed, and then fractionated with digitonin. Because the mitochondria isolated by centrifugal filtration were contaminated by adherent medium, the amount of \(^{14}\text{C}\)pyridoxal-P recovered was 23-37% higher than that found in mitochondria isolated by centrifugation and repeated washing (Table II). At both 15 and 60 min, the intermembrane space contained the largest quantity of \(^{14}\text{C}\) pyridoxal-P. However, the greatest net increase in \(^{14}\text{C}\)pyridoxal-P between these two time points took place in the

![Fig. 1. Uptake of \(^{14}\text{C}\)pyridoxal-P (PLP) and \(^{14}\text{C}\)pyridoxine (PN) by isolated rat liver mitochondria as a function of time. Conditions of incubation were described under "Experimental Procedures." Uptake was determined by centrifugal filtration. The concentrations of \(^{14}\text{C}\)pyridoxal-P and \(^{14}\text{C}\)pyridoxine in the medium are indicated on the right side of the figure.](image1)

![Fig. 2. Uptake of \(^{14}\text{C}\)pyridoxal-P (PLP) and \(^{14}\text{C}\)pyridoxine (PN) by isolated rat liver mitochondria as a function of the concentration of the B6 vitamer in the medium. Conditions of incubation were described under "Experimental Procedures." The incubation time was 60 min. Uptake was determined by centrifugal filtration.](image2)
matrix compartment. These data indicate that pyridoxal-P can penetrate the inner membrane and be retained in the matrix compartment. They also suggest that the rapid phase of pyridoxal-P uptake demonstrated in Fig. 1 was largely due to transport into the intermembrane space, whereas the slow phase was principally accounted for by the uptake in the matrix space.

Binding of Pyridoxal-P to Mitochondrial Proteins—The accumulation of pyridoxal-P by liver mitochondria against an apparent concentration gradient in the absence of energy expenditure suggests protein binding of pyridoxal-P after it is transported into the organelle. To demonstrate such binding in both the intermembrane and matrix compartments, mitochondria which had been incubated with 3 μM [14C]pyridoxal-P for 60 min were centrifuged, washed, and fractionated with digitonin. Proteins from the two mitochondrial compartments were then chromatographed on a Sephadex G-25 column. As shown in Fig. 4, 38% of the [14C]pyridoxal-P in the intermembrane space and 46% in the matrix space were found to be protein-bound. The method we employed, though clearly showing binding of pyridoxal-P to mitochondrial proteins, could not be relied upon to give an accurate measurement of the extent of binding. A falsely low result is expected if dissociation occurs during the isolation and chromatography procedures.

**DISCUSSION**

In order to study the mitochondrial transport of pyridoxal-P *in vitro*, it is essential to use conditions that will prevent pyridoxal-P from being metabolized. In the experiments reported here, isolated liver mitochondria were incubated in the presence of 20 mM 2-oxoglutarate and 10 mM inorganic phosphate. 2-Oxoglutarate was employed as the substrate because this α-keto acid had been shown to prevent pyridoxal-P from being converted to pyridoxamine-P by aminotransferases (1). High phosphate concentration was also used in the incubation medium to inhibit the hydrolysis of pyridoxal-P to pyridoxal (1). Under these conditions, the present experiments demonstrate that pyridoxal-P transport into liver mitochondria is an accumulative process, whereas pyridoxine transport is not concentration-dependent and is most likely due to passive diffusion (Figs. 1 and 2). This finding for pyridoxine is consistent with several reports which indicate that pyridoxine transport is by diffusion in a number of mammalian membrane systems (5–9). However, it is noteworthy that active transport of pyridoxine in yeast (10) has been established, as has facilitated diffusion coupled with metabolic trapping in *Salmonella typhimurium* (11).
The majority of the mitochondrial uptake of pyridoxal-P is greater than 90% of the endogenous phosphorylated B_{6} vitamins is located in the matrix of rat liver mitochondria is difficult to reconcile with our current data which show that occurs in the intermembrane space. This discrepancy, however, can be explained by the fact that, in this study, we had measured only the flux of radiolabeled pyridoxal-P and had not followed the transport process to its equilibrium. Due to the obvious limitation of prolonged incubation of mitochondria, we did not attempt to document whether uptake into the matrix was maintained indefinitely until all protein binding sites for pyridoxal-P became saturated and exchange between transported and endogenous pyridoxal-P was complete. However, the evidence we provide here would suggest that, if incubation could be continued without gross distortion of the permeability characteristics of the mitochondrial membranes, distribution of pyridoxal-P between the intermembrane space and the matrix at equilibrium would be dictated by the amount of binding proteins and their affinity for pyridoxal-P in the two compartments. Since the matrix contains the majority of the mitochondrial proteins including almost all of the B_{6}-dependent enzymes, it is reasonable to assume that eventually most of the pyridoxal-P taken up by the mitochondria will be found in that compartment.

Although we have shown that free pyridoxal-P can be transported into isolated mitochondria, a more complex mechanism may be involved in the intact cell. Recently, rapid penetration of aspartate aminotransferase into isolated rat liver mitochondria has been demonstrated (18, 19). It is thus conceivable that pyridoxal-P can be cotransported into the mitochondria with this and other B_{6}-dependent enzymes. Further studies are needed to elucidate the relative importance of these possible mechanisms in the mitochondrial transport of pyridoxal-P in vivo.

REFERENCES

Mitochondrial Vitamin B_{6} Transport

Since the mitochondrial transport of pyridoxal-P is not dependent on biological energy (Table I), the accumulation of this B_{6} vitamer is most likely due to protein binding (Fig. 4 and Table II). Pyridoxal-P in liver is extensively and avidly bound to proteins (12). It has been estimated that no more than 4-14 nmol of this B_{6} vitamer/g of tissue exists in a free or loosely bound form (12). The apparent gradient of pyridoxal-P accumulation decreases with increasing concentrations of this coenzyme in the medium. This inverse relationship between the apparent accumulation gradient and medium concentration is best explained by the saturation of high affinity binding sites at low pyridoxal-P concentrations, leaving only less effective sites operational at higher pyridoxal-P concentrations. It should be emphasized that, among the medium concentrations of pyridoxal-P studied, the 3 µM level is physiologically most relevant.

We have shown earlier (1) that isolated rat liver mitochondria lack pyridoxal kinase and pyridoxamine-P (pyridoxine-P) oxidase and cannot synthesize radiolabeled pyridoxal-P or pyridoxamine-P from their unphosphorylated precursors, including [^{14}C]pyridoxine, [^{3}H]pyridoxal, and [^{14}C]pyridoxine-P. However, when isolated rat hepatocytes were incubated in the presence of [^{14}C]pyridoxine, [^{14}C]pyridoxal-P was synthesized in the cytosol and eventually appeared in mitochondria isolated from these hepatocytes. These observations suggested that the uptake of radiolabeled pyridoxal-P by mitochondria was due to transport of newly synthesized [^{14}C]pyridoxal-P from the cytosol into mitochondria. The experiments here reported demonstrate that pyridoxal-P can be transported into isolated liver mitochondria and that it binds to proteins in both the intermembrane and matrix compartments (Table II and Fig. 4). Whereas the outer mitochondrial membrane is indiscriminately permeable to small molecules with a molecular weight of less than 5000, the inner membrane is quite selective in its transport of most charged and low molecular weight compounds (13). These general characteristics of mitochondrial membranes are compatible with the observed kinetics of [^{14}C]pyridoxal-P uptake shown in Fig. 1 and Table II. Specifically, with 3 µM radiolabeled pyridoxal-P, the early and rapid phase of uptake of this coenzyme most likely reflects transport across the outer membrane into the intermembrane space. By comparison, the later, slower, and more sustained phase of uptake can be explained by transport across the inner membrane into the matrix compartment. As shown in Table II, the rate of uptake of radiolabeled pyridoxal-P into the matrix is in the range of 0-16 pmol/h/mg of protein. This rate of uptake is consistent with slow diffusion across the inner membrane because the rate of active carrier-mediated mitochondrial transport of most anions is several orders of magnitude faster, in the range of nmol/min/mg of protein (13, 14). This conclusion agrees with previous observations that phosphorylated B_{6} compounds can slowly penetrate the membranes of red blood cells (15, 16), myelin (17), and isolated choroid plexus (6).

Upon initial inspection, the previous observation (1) that greater than 90% of the endogenous phosphorylated B_{6} vitamins is located in the matrix of rat liver mitochondria is difficult to reconcile with our current data which show that the majority of the mitochondrial uptake of pyridoxal-P occurs in the intermembrane space. This discrepancy, however, can be explained by the fact that, in this study, we had measured only the flux of radiolabeled pyridoxal-P and had